Type I IL-1 Receptor Mediates IL-1 and Intracellular IL-1 Receptor Antagonist Effects in Skin Inflammation

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The IL-1 system plays a key role in skin physiology and pathology. In this study, we used mutant mice lacking the type I IL-1 receptor (IL-1RI), lacking IL-1 receptor antagonist (IL-1Ra), or overexpressing the human intracellular (ic) IL-1Ra1 isoform, as well as combinations thereof, to dissect the role of the IL-1 system in phorbol 13-myristate 12-acetate (PMA)-induced skin inflammation. In wild-type (WT) mice, PMA application induced epidermal thickening and dermal inflammation. Skin IL-1α production and circulating levels of the acute-phase protein serum amyloid A (SAA) were elevated. In mice lacking IL-1RI or overexpressing icIL-1Ra1, PMA induced similar epidermal thickening as in WT mice, but dermal inflammation was partially prevented. Skin IL-1α mRNA expression was similar in PMA-treated IL-1RI−/− and WT mice, whereas the increase in serum SAA was suppressed in IL-1RI−/− mice. Interestingly, PMA-induced IL-1α mRNA expression was further enhanced by icIL-1Ra1 overexpression in an IL-1RI-dependent manner. Finally, IL-1Ra−/− mice spontaneously displayed skin lesions characterized by high IL-1β, but not IL-1α, expression. In conclusion, PMA-induced epidermal thickening and skin IL-1α expression were independent of IL-1 signaling, in contrast to dermal inflammation and systemic inflammatory response.

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INTRODUCTION

IL1 activity is mediated by two cytokines, IL-1α and IL-1β, which are produced by two different genes and share 26% amino-acid homology (see Dinarello (1996) for a review). IL-1β is primarily produced by monocytes and macrophages and is secreted after cleavage of its proform by IL-1β-convertase enzyme (also termed 'caspase-1'). IL-1α is synthesized as a 31-kDa precursor peptide, which is specifically cleaved by calpain-like proteases to generate C-terminal mature 17 kDa IL-1α and a N-terminal 16 kDa propeptide. Although pre-IL-1β is biologically inactive, both precursor and mature forms of IL-1α are able to bind to IL-1 receptors and activate intracellular signaling. IL-1 treatment of cells induces the formation of a high-affinity complex containing the type I IL-1 receptor (IL-1RI) and IL-1 receptor accessory protein (IL-1RAcP). The intracellular domains of IL-1RI and IL-1 receptor accessory protein then recruit the adapter protein MyD88 and IL-1 receptor-associated kinases, which are the proximal mediators of IL-1 signaling (O'Neill, 2002).

IL-1 receptor antagonist (IL-1Ra) is a natural inhibitor of IL-1 activity. IL-1Ra binds to cell surface IL-1 receptors, but does not recruit IL-1RAcP and thus does not induce any intracellular response (see Arend *et al.* (1998) for a review). IL-1Ra prevents the interaction between IL-1 and its receptors, and thus competitively inhibits its biological effects. IL-1Ra is produced as four different isoforms, one secreted (sIL-1Ra) and three intracellular (icIL-1Ra1, 2, 3), derived from the same gene (Arend *et al.*, 1998). The biological function of intracellular variants of IL-1Ra remains unclear. Intracellular IL-1Ra isoforms are able to prevent the binding of IL-1 to its cell-surface receptors. However, owing to their intracellular localization, icIL-1Ra isoforms cannot interact with cell surface IL-1 receptors unless released by dying cells or upon appropriate cellular activation (Lee *et al.*, 1997; Levine *et al.*, 1997; Wilson *et al.*, 2004). In addition, several *in vitro* studies using different types of epithelial cells and dermal fibroblasts have suggested that icIL-1Ra1 carries out specific anti-inflammatory functions inside cells, independently of IL-1RI binding (Watson *et al.*, 1995; Garat and Arend, 2003; Banda *et al.*, 2005; Kanangat *et al.*, 2006). However, these observations have not been confirmed in other studies (Muzio *et al.*, 1999; Evans *et al.*, 2006). In addition, no IL-1RI-independent effect of icIL-1Ra isoforms has been documented *in vivo* so far. In contrast, deletion of the IL-1Ra and IL-1RI genes proved to be epistatic for various phenotypes analyzed in knockout mice, indicating that all examined functions of IL-1Ra depended on the presence of a functional receptor (Irikura *et al.*, 2002).

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Abbreviations: ANOVA, analysis of variance; icIL-1Ra1, intracellular IL-1 receptor antagonist type I; IL-1RI, type I IL-1 receptor; IL-1Ra, IL-1Ra receptor antagonist; IL-1RAcP, IL-1 receptor accessory protein; MEF, mouse embryonic fibroblast; MIF, macrophage migration inhibitory factor; PMA, phorbol 13-myristate 12-acetate; SAA, serum amyloid A; tg, transgenic; WT, wild type

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Keratinocytes constitutively produce high amounts of IL-1α and the epidermis contains important quantities of biologically active preformed IL-1α (Murphy *et al.*, 2000). In addition, human, but not mouse, keratinocytes also produce limited amounts of IL-1β in inflammatory conditions (Ansel *et al.*, 1988; Zepter *et al.*, 1997). Furthermore, keratinocytes produce high amounts of icIL-1Ra1 isoform (Bigler *et al.*, 1992), as well as IL-1RI and the type II, non-signal transducing IL-1 receptor. All elements of IL-1 system are thus represented in the epidermis and IL-1 is believed to be centrally involved in cutaneous physiology and pathology (Murphy *et al.*, 2000). Deregulation of IL-1 system has been suggested to play a role in inflammatory skin diseases. Indeed, overexpression of IL-1α in the epidermis causes spontaneous skin disease characterized by hair loss, scaling, and focal inflammatory skin lesions (Groves *et al.*, 1995). Moreover BALB/c designates a mouse genetic background IL-1Ra −/− mice develop inflammation of the skin of the ear pinna with many features resembling psoriasis (Shepherd *et al.*, 2004).

The application of phorbol 13-myristate 12-acetate (PMA) to mouse skin results in an acute inflammatory response, including epidermal hyperplasia, abnormal keratinocyte differentiation, alteration of keratin expression, increased IL-1α production, and leukocyte infiltration (Reynolds *et al.*, 1998). Experiments with blocking antibodies have implicated IL-1α as a mediator of inflammatory cell infiltration and epidermal hyperplasia induced by PMA treatment (Lee *et al.*, 1994). In this study, we wanted to further dissect the role of the IL-1 system in response to PMA skin painting using mutant mice lacking IL-1RI or IL-1Ra, or overexpressing the human icIL-1Ra1 isoform. Furthermore, as contradictory findings have been reported concerning the existence of intracellular effects of icIL-1Ra1 in different cell types *in vitro*, we used icIL-1Ra1 transgenic (tg) mice lacking both endogenous IL-1Ra isoforms and the IL-1RI to specifically investigate potential IL-1RI-independent effects of icIL-1Ra1 in a model of skin inflammation *in vivo*. Finally, we also examined the effects of icIL-1Ra1 overexpression on cytokine production in different cell types *in vitro*.

RESULTS

We first assessed the cutaneous inflammatory response to three repeated applications of PMA on the back of wild-type (WT) DBA/1 mice. Two days after the last application of PMA, skin histology showed a marked epidermal thickening and significantly increased infiltration of inflammatory cells, mainly polymorphonuclear cells, into the dermis (Figure 1a and b). We examined the local expression of various cytokines in acetone or PMA-treated skin of WT mice by RNase protection assay. Among the cytokines tested, we observed strong expression of IL-1α, IL-1Ra, IL-18, and macrophage migration inhibitory factor (MIF) in PMA-treated WT skin and lower levels of IL-1β and IL-6. Although mRNA expression tended to be higher in PMA-treated skin for all cytokines, only IL-1α and IL-1Ra mRNA levels were significantly increased (Figure 1c). MIF levels were high in the skin of acetone-treated WT mice and not significantly increased by

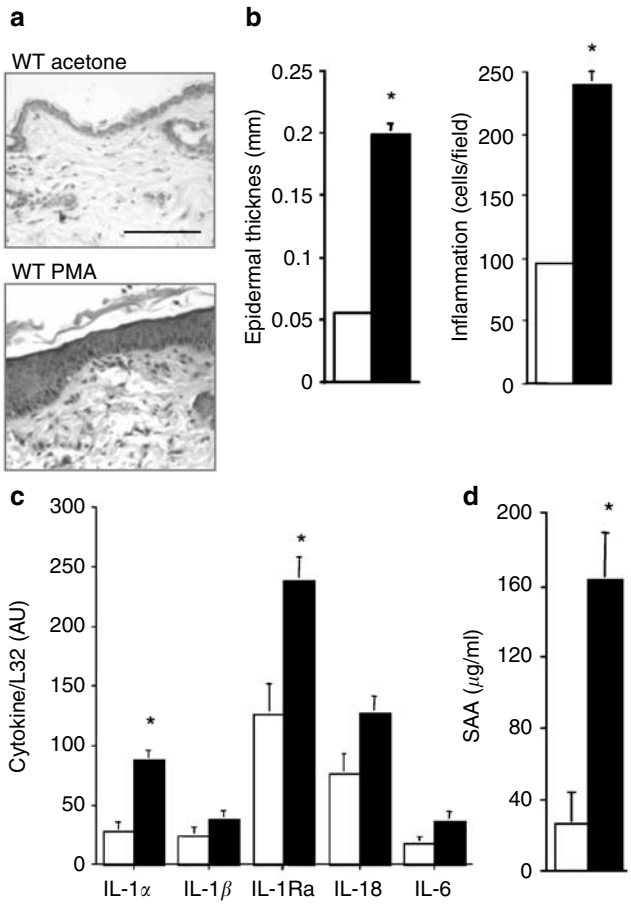

Figure 1. PMA-induced skin inflammation in WT mice. (**a**) Representative histological sections of acetone (upper panel, original magnification ×20) and PMA-treated (lower panel, original magnification ×20, scale bar = 500 μm) skin of WT mice are shown. PMA treatment resulted in marked epidermal thickening and increased infiltration of inflammatory cells into the dermis. (**b**) Results of histological scoring for epidermal thickness (left panel) and dermal inflammatory cell infiltration (right panel) are shown for acetone (white columns; $n=5$) and PMA-treated (black columns; $n=8$) WT mice. Results shown represent the mean ± SEM for each group of mice. *$P<0.05$ versus acetone-treated WT mice, as assessed by *t*-test. PMA painting induced a significant increase in both epidermal thickness and inflammatory cell infiltration. (**c**) Cytokine mRNA expression in the skin was analyzed by RNase protection assay and quantified by phosphorimaging for acetone (white columns; $n=3$) and PMA-treated (black columns; $n=8$) WT mice. Results are expressed as the ratio of cytokine over L32 mRNA expression. Results shown represent the mean ± SEM for each group of mice. *$P<0.05$ versus acetone-treated WT mice, as assessed by *t*-test. AU, arbitrary units. IL-1α and IL-1Ra mRNA expression was significantly increased in PMA-treated WT mice. (**d**) Circulating SAA levels were assessed by ELISA in the serum of acetone (white columns; $n=5$) or PMA-treated (black columns; $n=8$) WT mice. Results shown represent the mean ± SEM for each group of mice. *$P<0.05$ versus acetone-treated WT mice, as assessed by *t*-test. Serum SAA was significantly elevated in PMA-treated WT mice.

PMA treatment (WT acetone, $n=3$: 341 ± 31 arbitrary units (AU); WT PMA, $n=8$: 431 ± 44, AU; $P>0.05$). We also evaluated the systemic response to PMA treatment by measuring serum amyloid A (SAA) and IL-6 levels as markers of the acute-phase response. PMA application significantly increased SAA levels (Figure 1d), but circulating IL-6 levels

were below the limit of detection of the ELISA in both PMA and acetone-treated WT mice.

PMA-induced skin inflammation in absence of IL-1 activity

We bred various mutant mouse lines either lacking IL-1RI or overexpressing the human icIL-1Ra1 isoform in presence or absence of endogenous IL-1Ra. These mutant mice were used to investigate the response to PMA skin painting in absence of IL-1 activity. In addition, we used icIL-1Ra1 tg mice lacking both endogenous IL-1Ra isoforms and the IL-RI to investigate potential IL-1RI-independent effects of icIL-1Ra1. Histological analysis of PMA-treated skin indicated that epidermal thickness was similar in all groups of mice (Figure 2a), whereas inflammatory cell infiltration into the dermis was partially prevented in mice overexpressing icIL-1Ra1 and/or lacking IL-1RI, in presence or in absence of endogenous IL-1Ra (Figure 2b). Dermal cell infiltration (mean±SEM) was: 239±11 in WT ($n=8$), 178±5 in IL-1RI −/− ($n=5$, $P=0.001$ versus WT), 201±12 in icIL-1Ra1 tg ($n=4$, $P=0.0473$ versus WT), 154±4 in IL-1Ra −/− × icIL-1Ra1 tg ($n=5$, $P<0.0001$ versus WT), 150±4 in IL-1Ra −/− × IL-1RI−/− ($n=5$, $P<0.0001$ versus WT) and 160±2 in IL-1Ra−/− × IL-1RI−/− × icIL-1Ra1 tg mice ($n=8$, $P<0.0001$ versus WT). The effect of PMA on epidermal thickening is thus most likely direct, rather than IL-1 mediated, whereas inflammatory cell infiltration is partly IL-1 dependent.

Skin IL-1α mRNA expression was similarly elevated after PMA treatment in IL-1RI−/− mice, in presence or absence of endogenous IL-1Ra, and in WT mice (Figure 2c). This increase thus likely reflects a direct effect of PMA on keratinocytes and is not IL-1RI-signaling dependent. Interestingly IL-1α mRNA expression was significantly higher in PMA-treated skin of mice overexpressing icIL-1Ra1, in presence and in absence of endogenous IL-1Ra, as compared with the skin of PMA-treated WT mice. This effect of icIL-1Ra1 overexpression was dependent on the presence of IL-1RI, as it was not observed in IL-1Ra−/− × IL-1RI−/− × icIL-1Ra1 tg mice (Figure 2c). Expression of endogenous mouse (m) IL-1Ra (mIL-1Ra) mRNA was similar in PMA-treated skin of WT, icIL-1Ra1 tg, and IL-1RI−/− mice, whereas single and combined IL-1Ra−/− mutants obviously lacked mIL-1Ra mRNA (Figure 4c and data not shown). The PMA-induced increase in serum SAA levels was abolished in mice overexpressing icIL-1Ra1, deficient for IL-RI, or both, even

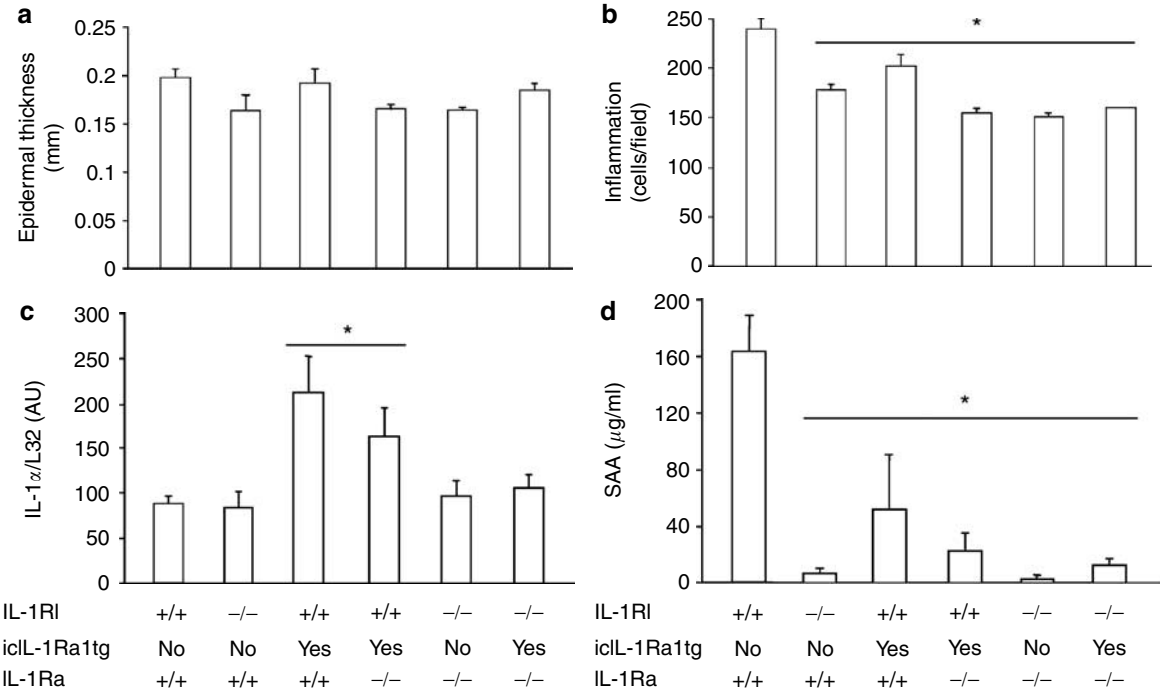

Figure 2. PMA-induced skin inflammation in absence of IL-1 activity. Results of histological scoring for (**a**) epidermal thickness and (**b**) dermal inflammatory cell infiltration are shown for PMA-treated WT ($n=8$), IL-1RI−/− ($n=5$), icIL-1Ra1 tg ($n=4$), IL-1Ra−/− × icIL-1Ra1 tg ($n=5$), IL-1Ra−/− × IL-1RI−/− ($n=5$) and IL-1Ra−/− × IL-1RI−/− × icIL-1Ra1 tg mice ($n=8$). Results shown represent the mean±SEM for each group of mice. *$P<0.05$ versus WT mice, as assessed by analysis of variance (ANOVA). Epidermal thickness was similar in all groups of mice, whereas inflammatory cell infiltration was significantly decreased in mice lacking IL-1RI and/or overexpressing icIL-1Ra1, in presence or in absence of endogenous IL-1Ra. (**c**) IL-1α mRNA expression was analyzed by RNase protection assay and quantified by phosphorimaging in PMA-treated WT ($n=8$), IL-1RI−/− ($n=5$), icIL-1Ra1 tg ($n=4$), IL-1Ra−/− × icIL-1Ra1 tg ($n=5$), IL-1Ra−/− × IL-1RI−/− ($n=5$), and IL-1Ra−/− × IL-1RI−/− × icIL-1Ra1 tg mice ($n=8$). Results are expressed as the ratio of IL-1α over L32 mRNA expression. Results shown represent the mean±SEM for each group of mice. *$P<0.05$ versus WT mice, as assessed by ANOVA. AU, arbitrary units. IL-1α mRNA levels were significantly elevated in icIL-1Ra1 tg and in IL-1Ra−/− × icIL-1Ra1 tg, as compared with WT mice. (**d**) Circulating SAA levels were assessed by ELISA in the serum of PMA-treated WT ($n=8$), IL-1RI−/− ($n=5$), icIL-1Ra1 tg ($n=4$), IL-1Ra−/− × icIL-1Ra1 tg ($n=5$), IL-1Ra−/− × IL-1RI−/− ($n=5$) and IL-1Ra−/− × IL-1RI−/− × icIL-1Ra1 tg mice ($n=8$). Results shown represent the mean±SEM for each group of mice. *$P<0.05$ versus WT mice, as assessed by ANOVA. SAA levels were significantly lower in PMA-treated mice lacking IL-1RI and/or overexpressing icIL-1Ra1, in presence or in absence of endogenous IL-1Ra.

in the absence of endogenous IL-1Ra, indicating that the systemic inflammatory response to PMA treatment of the skin is critically dependent on IL-1 signaling (Figure 2d).

Skin phenotype of IL-1Ra−/− mutant mice

We next examined the phenotype of IL-1Ra−/− mice, where IL-1 activity is not counterbalanced by IL-1Ra. The IL-1Ra−/− mice used in this study lack all IL-1Ra isoforms. These mice were backcrossed for six generations or more into DBA/1 background. They were bred and housed in our conventional animal facility and displayed a general phenotype very similar to that previously reported for IL-1Ra−/− mice in the C57BL/6 background (Irikura *et al.*, 2002). In particular, we observed decreased fertility, a general appearance suggesting illness, which included runting, piloerection, and mild weight loss, as well as, in some cases, moderate to severe respiratory distress. Although young adult IL-1Ra−/− mice were generally asymptomatic, this illness phenotype increasingly appeared with aging, and from 4 to 6 months of age, all mice displayed at least some of these symptoms. Crossing IL-1Ra−/− mice with icIL-1Ra1 tg and/or IL-1RI−/− mice completely reverted this general phenotype, confirming the epistasis between IL-1Ra and IL-1RI, which had been previously reported (Irikura *et al.*, 2002). As in this previous report, we failed to identify a specific defect to explain the ill health observed in IL-1Ra−/− mice, which is likely to be the consequence of a chronic inflammatory state, caused by inadequate perpetuation and amplification of various inflammatory processes occurring in these mice throughout their lives.

IL-1Ra−/− mice displayed a macroscopic skin phenotype similar to that previously described in mice overexpressing IL-1α in the skin (Groves *et al.*, 1995). They had sparse hair, particularly in the neck region and at the base of the tail, and some IL-1Ra−/− mice exhibited scaling and focal inflammatory skin lesions, mostly situated on the back. These lesions appeared with aging and were generally more pronounced in old (>4–6 months) mice. As for their general ill-health phenotype, crossing of IL-1Ra−/− mice with icIL-1Ra1 tg and/or IL-1RI−/− mice completely abolished the apparition of the skin phenotype. Although some control IL-1Ra−/− mice displayed normal skin histology (Figure 3a, upper left panel), marked spontaneous epidermal thickening, accompanied by increased dermal inflammatory cell infiltration, could be observed in skin sections of about 40% of the IL-1Ra−/− mice (Figure 3a, upper right panel and Figure 3b). However PMA-treated IL-1Ra−/− mice displayed epidermal thickening and inflammatory cell infiltration, indistinguishable from that observed in PMA-treated WT mice (Figure 3a, lower panel and Figure 3b).

IL-1α mRNA expression was similar in acetone-treated skin of WT and IL-1Ra−/− mice, and comparably increased upon PMA treatment for both genotypes (Figure 4a). Parallel changes were also observed for IL-1α protein expression in the skin of WT and IL-1Ra−/− mice (Table 1). By immunohistochemistry, high IL-1α expression was detected in particular in the epidermis of PMA-treated WT and IL-1Ra−/− mice (Figure 4b). Interestingly, we detected increased IL-1β mRNA expression in the skin of IL-1Ra−/− knockout mice

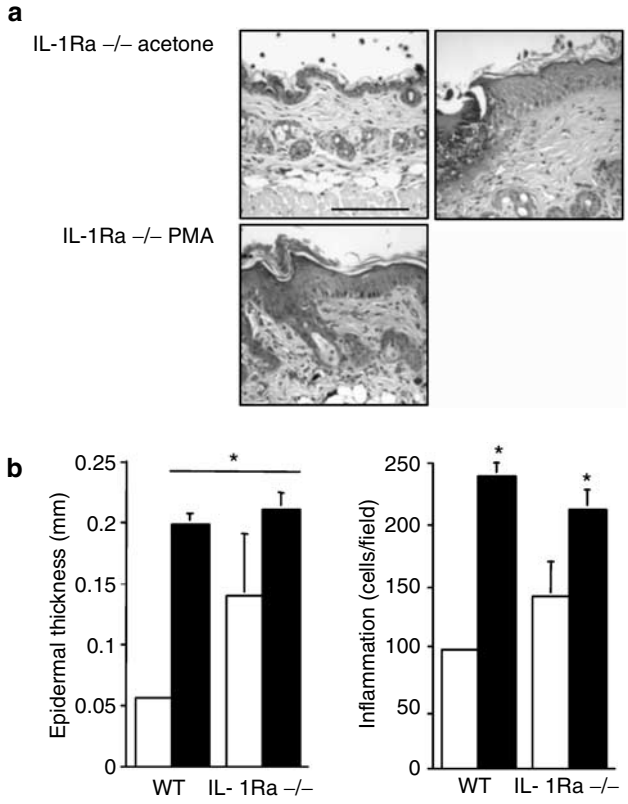

Figure 3. Skin phenotype in acetone and PMA-treated IL-1Ra−/− mice. (**a**) Representative histological sections of acetone (upper panels, original magnification ×20, bar = 500 μm) and PMA-treated (lower panel, original magnification ×20) skin of IL-1Ra−/− mice are shown. Although some control IL-1Ra−/− mice displayed normal skin histology (upper left panel), marked spontaneous epidermal thickening, accompanied by inflammatory cell infiltration into the dermis, could be observed in about 40% of the IL-1Ra−/− mice (upper right panel). PMA-treated IL-1Ra−/− mice displayed epidermal thickening and dermal inflammatory cell infiltration similar to that observed in PMA-treated WT mice. (**b**) Results of histological scoring for epidermal thickness (left panel) or inflammatory cell infiltration (right panel) are shown for acetone (white columns; $n = 5$ per genotype) or PMA-treated (black columns; $n = 8$ per genotype) WT and IL-1Ra−/− mice. Results shown represent the mean ± SEM for each group of mice. *$P < 0.05$ versus acetone-treated WT mice, as assessed by ANOVA. IL-1Ra−/− mice spontaneously displayed an increased epidermal thickness and a trend for higher inflammatory cell infiltration into the dermis. PMA painting resulted in similar epidermal thickening and dermal inflammatory cell infiltration in WT and IL-1Ra−/− mice.

(Figure 4c). IL-1β mRNA expression was variable and reached remarkably high levels in some mice. High IL-1β mRNA expression correlated with the presence of macroscopic skin lesions (Figure 4c, right panel). In contrast to IL-1α, IL-1β mRNA expression was not significantly affected by PMA treatment in either WT or IL-1Ra−/− mice (Figure 4a and c). IL-1β protein levels paralleled IL-1β mRNA expression in the skin of WT and IL-1Ra−/− mice (Table 1). Again, we observed a high inter-individual variability in IL-1β protein expression levels, which was highest in IL-1Ra−/− mice displaying important macroscopic skin lesions.

Consistently with their general ill health and skin phenotype, IL-1Ra−/− mice had spontaneously elevated serum

SAA levels (Figure 4d). Although PMA application significantly increased SAA levels in WT mice, values in IL-1Ra−/− mice were not further enhanced. Serum IL-6 levels remained below the detection limit of the test in all the different WT and mutant mice, except in some acetone or PMA-treated IL-1Ra−/− mice, where serum IL-6 ranged from 18 to 375 pg/ml. Highest IL-6 levels were observed in old IL-1Ra−/− mice displaying a marked general illness phenotype and spontaneous macroscopic skin lesions, independently of PMA treatment.

Table 1. IL-1α and IL-1β protein levels in skin lysates

Genotype	Treatment	IL-1α (ng/mg protein)[1]	IL-1β (pg/mg protein)[1]
WT	Acetone	5.1±0.4	16±3
	PMA	10.9±2.6[2]	28±3
IL-Ra−/−	Acetone	1.7±0.7	511±295
	PMA	7.1±1.05[2]	310±242

[1]Cytokine levels are shown for acetone (n=3) or PMA-treated (n=3) WT mice, and acetone (n=4) or PMA-treated (n=4) IL-1Ra−/− mice. Results shown represent the mean±SEM for each group of mice.
[2]$P<0.05$ versus acetone-treated mice of same genotype, as assessed by t-test.

DISCUSSION

We characterized the effects of repeated applications of PMA onto the skin of WT and mutant DBA/1 mice. In WT mice, we observed epidermal hyperplasia, inflammatory cell infiltration into the dermis, and increased expression of IL-1α and IL-1Ra in the skin, as previously reported after a single application of PMA in different strains of mice (Oberyszyn *et al.*, 1993; Lee *et al.*, 1994; Reynolds *et al.*, 1998; La *et al.*, 1999). We also observed a trend toward increased expression of other cytokines such as IL-18, IL-6, and MIF. IL-1β expression was rather low. Finally, repeated PMA treatment of the skin also induced a systemic inflammatory response, as indicated by increased circulating levels of SAA.

We then examined the response to PMA skin painting in absence of IL-1 signaling. IL-1RI−/− mice have been characterized previously for their lack of responsiveness to

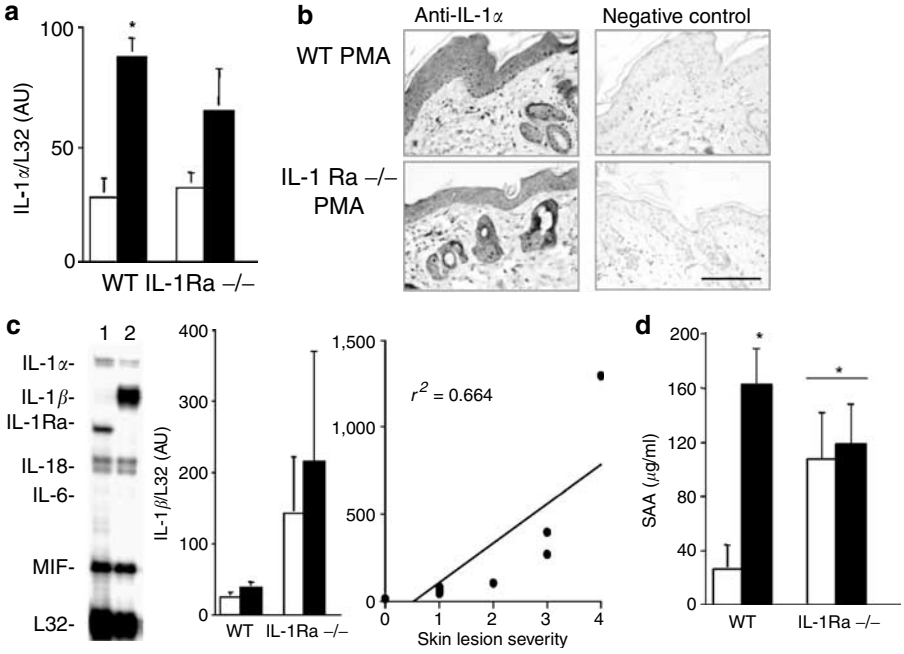

Figure 4. Skin IL-1α and IL-1β mRNA expression and systemic inflammatory response in IL-1Ra−/− mice. (**a**) IL-1α mRNA levels were quantified by RNase protection assay for acetone (white columns; WT, n=3; IL-1Ra−/−, n=5) or PMA-treated (black columns; n=8 per genotype) WT and IL-1Ra−/− mice. Results shown represent the mean±SEM for each group of mice. *$P<0.05$ versus acetone-treated WT mice, as assessed by ANOVA. AU, arbitrary units. PMA painting resulted in similar changes in IL-1α mRNA expression in WT and IL-1Ra−/− mice. (**b**) IL-1α protein expression was examined by immunohistochemistry in PMA-treated skin of WT (upper left panel, original magnification ×20) and IL-1Ra−/− mice (lower left panel, original magnification ×20). Incubations without the first antibody are shown as negative controls (right panels, original magnification ×20, bar=500 μm). High IL-1α expression was observed in particular in the epidermis. (**c**) Left panel: a representative RNase protection assay is shown for skin RNA isolated from a PMA-treated WT mouse (lane 1) and an acetone-treated IL-1Ra −/− mouse (lane 2). The identity of the detected bands is indicated on the left. Middle panel: IL-1β mRNA levels were quantified by RNase protection assay for acetone (white columns; WT, n=3; IL-1Ra−/−, n=5) or PMA-treated (black columns; n=8 per genotype) WT and IL-1Ra−/− mice. Results shown represent the mean±SEM for each group of mice. AU, arbitrary units. Right panel: IL-1β mRNA levels correlated with the severity of macroscopic skin lesions observed on IL-1Ra−/− mice (n=12, P=0.0015 as assessed by Spearman's correlation). The graph shows a linear regression of IL-1β mRNA expression levels as a function of macroscopic skin lesion scores (r^2=0.664). (**d**) Circulating SAA levels were assessed by ELISA in the serum of acetone (white columns; n=5 per genotype) or PMA-treated (black columns; n=8 per genotype) WT and IL-1Ra−/− mice. Results shown represent the mean±SEM for each group of mice. *$P<0.05$ versus acetone-treated WT mice, as assessed by ANOVA. SAA was significantly increased in acetone-treated IL-1Ra−/− as compared with WT mice. SAA levels were similar in PMA-treated WT and IL-1Ra−/− mice.

IL-1 (Glaccum *et al.*, 1997), which we further confirmed using *in vitro* cultures of mouse embryonic fibroblast isolated from these mice. Similarly, icIL-1Ra1 tg mice express the human icIL-1Ra1 from a strong ubiquitous promoter, which results in overexpression in all tissues and cell types examined, including keratinocytes and dermal fibroblasts, as well as in high circulating levels of hIL-1Ra1 in the serum (Gabay *et al.*, 2001; Palmer *et al.*, 2003). These mice are completely resistant to collagen-induced arthritis, an experimental model of immune-mediated arthritis which is strongly IL-1 dependent (Palmer *et al.*, 2003). Using mutant mice lacking IL-1RI or overexpressing icIL-1Ra1, we observed that epidermal thickening and IL-1α expression induced by repeated PMA treatment of the skin are independent of IL-1 signaling, in contrast to dermal inflammation and systemic inflammatory response, which are respectively partially and completely inhibited in absence of IL-1 activity. The effect of PMA on IL-1α expression in the epidermis likely reflects a direct effect of this compound on keratinocytes (Lee *et al.*, 1993), which we also observed in keratinocyte cultures *in vitro*. In addition to IL-1α, PMA may also directly induce the production of other cytokines and chemokines, including tumor necrosis factor-α, monocyte chemoattractant protein-1, and macrophage inflammatory protein-2 (Jamieson *et al.*, 2005; Murakawa *et al.*, 2006), which are likely to contribute to inflammatory cell infiltration into the dermis.

Our findings are somewhat different from previously reported observations, which indicated that local or systemic treatment of mice with anti-IL-1α antibodies, as well as overexpression of the inhibitory type II IL-1 receptor in keratinocytes, blocked both PMA-induced epidermal thickening and skin inflammation (Oberyszyn *et al.*, 1993; Lee *et al.*, 1994; Rauschmayr *et al.*, 1997). The studies using anti-IL-1α antibodies examined only the acute inflammation induced by a single application of PMA, and this difference in the experimental protocol might account for differences observed. We used, indeed, a more stringent model of multiple PMA applications (Jamieson *et al.*, 2005) and it has been observed previously that some pharmacological agents, which are active in the acute PMA model, are not efficient in multiple application models (Stanley *et al.*, 1991). In a preliminary experiment, we compared the effect of a single PMA application on WT and icIL-1Ra1 tg mice. We observed marked epidermal thickening in WT mice 48 hours after a single application of PMA. Epidermal thickening was also observed in icIL-1Ra1 tg mice, although it appeared reduced as compared with WT mice, suggesting that the effect of a single PMA application is indeed partially prevented in icIL-1Ra 1 tg mice. However, this observation is still different from the results reported by Lee *et al.* (1994), who showed that epidermal thickening induced by a single PMA application was totally inhibited by injection of anti-IL-1α antibodies. It thus has to be stressed that the difference in the experimental protocols used is only one potential explanation for the discrepancies observed between different studies. Indeed, overexpression of type II IL-1 receptor was also reported to decrease epidermal thickening after multiple PMA applications (Rauschmayr *et al.*, 1997), suggesting that additional factors, such as for instance the use of different mouse strains and different genetic backgrounds, might influence the results obtained.

IL-1α mRNA expression was significantly higher in PMA-treated skin of icIL-1Ra1 overexpressing, as compared with WT mice and this effect was dependent on the presence of IL-1RI. Although the exact mechanism underlying this effect is not clear, this observation is consistent with a recent study describing mutual upregulation of IL-1α and IL-1Ra expression in mouse keratinocytes, in which IL-1α release was found to be increased several folds in epidermal sheets derived from IL-1Ra tg epidermis (Mee *et al.*, 2005).

Altogether, the effects of icIL-1Ra1 overexpression, which we observed in this model of PMA-induced skin inflammation, were either similar to those observed upon deletion of IL-1RI or IL-1RI dependent. These effects are thus likely mediated by binding of extracellular icIL-1Ra1, which circulates at high levels in icIL-1Ra1 tg mice (Palmer *et al.*, 2003), to IL-1RI. In addition, as previously described in other models (Irikura *et al.*, 2002), the effects of IL-1Ra and IL-RI deletion on both the general and the cutaneous phenotype were epistatic, suggesting that all effects of IL-1Ra are mediated by IL-1RI. We thus failed to observe any IL-1RI-independent effects of icIL-1Ra1 in skin inflammation *in vivo*. However, given the existence of previous reports describing intracellular anti-inflammatory activity of icIL-1Ra 1 in human keratinocyte cell lines and dermal fibroblasts (Banda *et al.*, 2005; Kanangat *et al.*, 2006), we further investigated potential effects of icIL-1Ra1 *in vitro* using cultures of various cell types derived from our mutant mice, including keratinocytes, dermal fibroblasts, embryonic fibroblasts, and bone marrow-derived macrophages (data not shown). We could not observe any anti-inflammatory effect of icIL-1Ra1 overexpression on cytokine production in the different cell types examined. There are some differences in the experimental systems and readouts used between our experiments and previous studies in human cells and the results obtained are thus not directly comparable. Nevertheless, our observations argue against an anti-inflammatory effect of intracellular IL-1Ra in mouse primary cell cultures.

DBA/1 IL-1Ra−/− mice displayed a spontaneous skin phenotype very similar to the one described for mice overexpressing IL-1α in the epidermis (Groves *et al.*, 1995). In contrast, their ears appeared normal, and their skin lesions differed from the psoriasis-like modifications described on the ears of IL-1Ra−/− mice bred into the BALB/c background. These observations further emphasize the strong background dependence of the different inflammatory diseases that have been described in IL-1Ra−/− mice (Horai *et al.*, 2000; Nicklin *et al.*, 2000). Interestingly, although PMA treatment preferentially increased IL-1α expression, the spontaneous skin lesions of IL-1Ra−/− mice correlated with high IL-1β expression, suggesting that these two homologous cytokines play distinct roles in different types of skin inflammation.

In conclusion, epidermal thickening and IL-1α expression induced by repeated PMA treatment of the skin were independent of IL-1 signaling, in contrast to dermal inflammation and systemic inflammatory response. We failed to detect any IL-1RI-independent effects of icIL-1Ra1 in this model of

skin inflammation. Finally, IL-1Ra−/− mice displayed spontaneous skin lesions characterized by high IL-1β expression.

MATERIALS AND METHODS

Mice

WT DBA/1 mice were obtained from Janvier (Le Genest St Isle, France). IL-1Ra−/− mice were originally obtained from Dr E Hirsch (Hirsch *et al.*, 1996) and backcrossed for more than six generations into the DBA/1 background. IL-1RI−/− mice backcrossed for five generations into the DBA/1 background were obtained from Amgen Inc. (Thousand Oaks, CA) (Glaccum *et al.*, 1997). Mice tg for the human icIL-1Ra1 isoform were described previously (Gabay *et al.*, 2001) and backcrossed for more than 10 generations into the DBA/1 background. IL-1Ra−/− and icIL-1Ra1 tg mice were crossed to obtain IL-1Ra−/− mice carrying the human icIL-1Ra1 transgene. IL-1Ra−/− × icIL1-Ra1 tg mice were bred with IL-1RI−/− mice to create IL-1Ra and IL-1RI double knockout mice, as well as IL-1Ra and IL-1RI double knockout mice carrying the human icIL-1Ra1 transgene. IL-1Ra−/−, IL-1RI−/−, and double knockout mice were genotyped by PCR using the following primer combinations: mIL-1Ra forward 5′-aaccagctcattgctgggtactta-3′ and mIL-1Ra reverse 5′-gcccaagaaca cactatgaaggtc-3′ for IL-1Ra; mIL-1RI forward 5′-gagttacccgaggtc cagtgg-3′, mIL-1RI reverse 5′-ccgaagaagctcacgttgtcaag-3′, and mIL-1RI neo 5′-gaatgggctgaccgcttcctcg-3′ in a three primer PCR reactions for IL-1RI. Expression of the human icIL-1Ra1 transgene was assessed by ELISA in serum samples, as described by Palmer *et al.* (2003). All single and double knockout mice used for the experiments described in this study were homozygous, whereas icIL-1Ra1 tg mice were hemizygous for the transgene. All mice were housed under conventional conditions, and water and standard laboratory chow were provided *ad libitum*. All animal experiments were approved by the Animal Ethics Committee of the Geneva University School of Medicine and by the Geneva Veterinarian Office.

PMA-induced skin inflammation

Male or female WT and mutant mice were used between 2 and 13 months of age. Their backs were shaved with electric clippers 3 days before experiments. PMA (Sigma, Buchs, Switzerland; 1 μg in 200 μl acetone), or acetone (200 μl) for control mice, was applied onto the dorsal surfaces of shaved mice. PMA, respectively acetone, application was repeated 24 and 48 hours later (Jamieson *et al.*, 2005). Mice were killed 48 hours after the last application. At killing, acetone or PMA-treated skin was dissected and cut into 2–3 small pieces. One piece was fixed in 10% buffered formalin for histological analysis and 1–2 pieces were immediately frozen in liquid nitrogen and stored at −80°C, until used for RNA or protein lysate preparation.

Scoring of macroscopic skin lesion

Macroscopic skin lesions observed in IL-1Ra−/− mice were scored on a 4-point scale, in which 0 = normal appearance, 1 = localized hair loss and scaling, 2 = extensive hair loss and scaling with presence of 1–2 inflammatory foci, 3 = extensive hair loss, scaling, and presence of scabs, and 4 = extensive hair loss, scabs, and open wounds.

Histology

Skin samples were fixed in 10% phosphate-buffered formaldehyde, embedded in paraffin, and processed for histological analysis. Sections were cut at 5 μm, mounted onto slides, and stained with hematoxylin and eosin according to the standard procedures.

Epidermal thickness measurements

Epidermal thickness of mice was measured with a Zeiss Axioskop 2 microscope using a graded ocular micrometer at × 40 magnification and multiplied by 10 to correct the scale. The evaluation was performed by one of the authors (GK) in a masked fashion. Five measurements were performed per mouse and the average value was calculated.

Analysis of dermal cellularity

Dermal cells were counted with a Zeiss Axioskop 2 microscope at × 40 magnification. The evaluation was performed by one of the authors (GK) in a masked fashion. Three counts were made per mouse and the average value was calculated.

RNase protection assay

Small pieces of the skin were homogenized in Trizol reagent (Gibco, Life Technologies AG, Basel, Switzerland) and total RNA was prepared according to the manufacturer's instructions. Expression levels of IL-12, IL-10, IL-1α, IL-1β, IL-1Ra, IL-18, IL-6, interferon-γ, MIF, L32 ribosomal protein, and glyceraldehyde-3-phosphate dehydrogenase mRNA were analyzed by RiboQuant RNase protection assay, using the mCK-2b multi-probe template set from BD Biosciences (Heidelberg, Germany). Briefly, riboprobes were ^{32}P-labeled and hybridized overnight in solution with 10 μg total RNA. The hybridized RNA was digested with RNases A and T1 and the remaining RNase-protected probes were purified, resolved on denaturing polyacrylamide gels, and imaged by autoradiography according to the RiboQuant protocol. The protected bands representing cytokine mRNA expression were quantified by phosphorimaging using a Cyclone Storage Phosphor System (PerkinElmer Life Sciences, Zaventem, Belgium) and normalized for L32 mRNA expression. Cytokines detectable in acetone and/or PMA-treated skin were IL-1α, IL-1β, IL-1Ra, IL-18, IL-6, and MIF.

Measurement of circulating SAA and IL-6 levels

Blood was taken at the end of experiment by cardiac puncture. Serum levels of SAA were determined using a direct ELISA, as described previously (Sipe *et al.*, 1989). The detection limit for this test is 13 μg/ml. Serum levels of IL-6 were measured using a commercial DuoSet ELISA Development System from R&D Systems (Abington, UK). The detection limit for this test is 39 pg/ml.

Measurement of IL-1α and IL-1β protein levels in skin extracts

Small pieces of the skin were homogenized in ice-cold lysis (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM phenylmethanesulfonyl fluoride, 0.5% Triton X-100) and the lysates were cleared by centrifugation at 13,000 r.p.m., 4°C for 15 minutes. Protein concentration in the lysates was assessed using the Biorad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). IL-1α and IL-1β concentrations were determined using commercial DuoSet ELISA Development Systems (R&D Systems, Abington, UK). Detection limit is 16 pg/ml for IL-1α and IL-1β.

Immunohistochemistry for IL-1α

IL-1α expression was detected by immunohistochemistry on paraffin-embedded sections. The sections were deparaffinized, rehydrated,

and boiled for 10 minutes in citrate buffer (10 mM, pH 6) in a microwave oven for heat-induced antigen retrieval. Endogenous peroxidase activity was blocked with 0.6% H_2O_2 in methanol:H_2O (1:1) for 10 minutes. Sections were permeabilized in phosphate-buffered saline containing 0.1% Triton X-100 for 15 minutes and blocked with phosphate-buffered saline containing 3% BSA and 10% normal goat serum for 1 hour at RT. Sections were then stained using a rabbit polyclonal anti-IL-1α antibody (sc-7929; Santa Cruz Biotechnologies, Santa Cruz, CA; 1/100) in phosphate-buffered saline containing 1.5% BSA and 5% normal goat serum overnight at 4°C. After washing in phosphate-buffered saline, bound antibody was visualized using a biotinylated goat anti-rabbit secondary antibody (DakoCytomation AG, Baar, Switzerland; 1/250), the avidin–biotin–peroxidase complex and 3,3′-diaminobenzidine (DakoCytomation) as a chromogen. Slides were counterstained with hemalun. An incubation without the first antibody served as a negative control.

Statistical analysis

Significance of differences was calculated by Student's unpaired *t*-test or analysis of variance (ANOVA) as appropriate. A difference between experimental groups was considered significant when the *P*-value was <0.05.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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